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Award Number: DAMD17-98-1-8193

TITLE: Engineering of Specific Tissue Inhibitors to Block ADAM Type Metalloprotease-Mediated Mammary Neoplasia

PRINCIPAL INVESTIGATOR: Yibing Yan, Ph.D.
Z. Werb, Ph.D.

CONTRACTING ORGANIZATION: University of California San Francisco
San Francisco, California 94143-0962

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE
Engineering of Specific Tissue Inhibitors to Block ADAM Type Metalloprotease-Mediated Mammary Neoplasia

5. FUNDING NUMBERS
DAMD17-98-1-8193

6. AUTHOR(S)
Yibing Yan, Ph.D.
Z. Werb, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California San Francisco
San Francisco, California 94143-0962

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Breast cancers arise from the mis-regulated cell fate during the life span of mammary epithelial cells in growth, differentiation and death cycle. Among many genes controlling the cell fate, Notch gene (Int3), when over-activated, invariably leads to development of mouse mammary tumor. In *Drosophila*, Notch activation requires proteolytic processing by Kuzbanian, a member of membrane bound metalloprotease ADAM family. But does mKUZ direct the activation of Notch pathway in mammalian cells? What is the effect of inhibition of mKUZ in neoplastic transformation? Here, we report that mouse Kuzbanian (mKUZ) induced the expression of HES transcription and p38MAP kinase phosphorylation, which both are the down stream events triggered by Notch in *Drosophila*. mKUZ resided in cytoplasm, concentrated on the peri-nuclear region and associated with the cytosekeleton, consistent with that Notch activation occurs in the cytoplasm. Furthermore, we show that expression of dominant negative mutant of mKUZ lead to down regulation of wild type mKUZ, and rendered cells resistant to MYC induced cell transformation. Therefore, targeting the matrix metalloprotease mKUZ may prevent early neoplasia.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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Contents

Note: This report contains unpublished data.

Introduction

Breast cancers arise from the mis-regulated cell fate during the life span of mammary epithelial cells in growth, differentiation and death cycle. Among many genes controlling cell fates, Notch gene (Int3), when over-activated, invariably leads to development of mouse mammary tumor. Notch activation, in turn, requires proteolytic processing by Kuzbanian, a member of membrane bound metalloprotease ADAM family. But does mKUZ indeed direct the activation of Notch pathway in mammalian cells? What is the effect of inhibition of mKUZ in neoplastic transformation? Here, we report that mouse Kuzbanian (mKUZ) induced the expression of HES transcription and p38MAP kinase phosphorylation, which both are the down stream events triggered by Notch in *Drosophila*. mKUZ resided primary in cytoplasm, concentrated on the peri-nuclear region and associated with the cytosekeleton, consistent with that Notchactivation occurs in the cytoplasm. Furthermore, we show that expression of dominant negative mutant of mKUZ lead to down regulation of wild type mKUZ, and rendered cells resistant to Myc induced cell transformation. Therefore, targeting the matrix metalloprotease mKUZ may prevent early neoplasia.

Body

mKUZ activated the Notch pathway in mammalian cells. Since the discovery of mKUZ, its role in activation of Notch pathway has been subjected to an intense debate. It is important to validate that the mKUZ is indeed involved in activation of Notch pathway in mammalian cells, and that inhibition of mKUZ could reduce Notch mediated neoplastic transformation. Notch activation directly causes the transcription of HES family of genes, which are, in turn, transcription suppressers. If mKUZ is involved in the Notch signaling pathway, then increase of mKUZ in the cells should lead to similar increase of HES transcription. This was indeed the case as shown in Figure 1 A. Luciferase activity under the control of HES transcription element raised dramatically when mKUZ was present. The dominant negative form of mKUZ (mDNK), which the catalytic domain is deleted, did not induce the HES transcription.

Notch function requires activation of Jun N-terminal Kinase (JNK) in addition to stimulating the expression of HES, as suggested by the genetic analysis in *Drosophila*. Transient transfected COS-7 cells were used to test if mKUZ plays a role in JNK and p38MAPK pathway. Preliminary data on cultured cells showed that expression of KUZ, but not DNK, lead to the increase of phosphorylated p38 MAP Kinase (Figure 1 B).

These results showed that mKUZ, like its *Drosophila* orthologue, did operate in Notch signaling pathway. Further test of its function in mammary tumor formation is currently underway.

mKUZ located in cytoplasm The ADAM family of metalloproteases are predicted to has single transmembrane domain, thus they should be displayed on the cell surface, yet there is no evidence in the literature to support this prediction. To address the localization of mKUZ, an epitope tag (HA or Flag tag sequence) was added to the carboxy-terminal of mKUZ amino acid sequence. The mKUZ and mDNK cDNA's were transfected into the COS-7 cell or RAT-1A cells, and their localizations were traced by immunofluorescence stain. The results were shown in Figure 2. In COS-7 cells, mKUZ were abundant in two locations: in the peri-nuclear area, and associated with cytoskeleton networks (Figure 2 B). On the other hand, the dominant negative mKUZ, which lacks the catalytic domain, resided in very different locations in cells (Figure 2 C). The results suggest that proteolysis by mKUZ might take place in the cytoplasmic membrane systems. In such system, mKUZ mediated cleavage might be able to efficiently couple the maturation and transportation processes of its substrates, such as Notch receptors or their membrane-bound ligands. Experiments are under way to test if TACE (TNF-alpha-Converting Enzyme), a closely related ADAM, has similar cellular localization.

Dominant negative mKUZ down-regulated the wild type mKUZ protein. The dominant negative mutant is a useful tool in elucidating the rule of wild type gene. In *Drosophila*, the dominant negative KUZ blocks the function of KUZ, creating a phenocopy of kuzbanian null mutant. In general, such dominant negative effect is attributed to sequestration of factors that interact with the gene product. Surprisingly, in mammalian cells, dominant negative mKUZ, which lacks catalytic domain of metalloprotease, lowered the level of mature mKUZ protein significantly as shown in Figure 3. Co-transfected with same amount of plasmids bearing same promoter, with or without an unrelated reporter protein GFP cDNA, did not affect the mKUZ protein level; therefore, it is less likely that such suppression occurs at the level of competing for transcription factors. The mDNK may interact with turnover (or processing) machinery for mKUZ in cells. Interestingly, mKUZ also expressed different amounts in cells with or without transforming SV40 large T-antigen (data not shown).

mKUZ interfered with the action of *myc* oncogene. To investigate potential benefit of inhibiting mKUZ in blocking early tumor formation, the effects of mKUZ and mDNK on

cell transformation were tested. In RAT1A cells expressing *myc*-ER chimeric protein, *myc* oncogene can be activated by adding 4-Hydroxytamoxifen. Activation of *myc* oncogene leads to cell transformation. Transformed cells did not form a monolayer like that of normal cells (Figure 4 A), because the loss of contact inhibition (Figure 4 B). But introducing mKUZ and mDNK in those cells could often change the transformation potentials of *myc* oncogene. For example (Figure 4 C to F), when mKUZ and mDNK were expressed together with the N-*myc*, mDNK rendered RAT1A cells resistant to the transformation (Figure 4 F). Whether or not such effects are due directly to the sequestration of endogenous KUZ by mDNK is currently under investigation; however, such experiment points out the possibility that signaling pathway regulating the cell fates can also operate in the cell transformation process leading to tumor formation.

Technical modification of proposed work. During the course of this work, several technical modifications seem necessary. The mKUZ cDNA turns out to be very unstable or toxic to most *E. Coli* strains for molecular cloning. In these strains, the mKUZ expressing plasmid grew very slow and was constantly modified by the hosts. After screen eight *E. Coli* strains, an *Epicurian Coli* strain SURE2 (invitrogen) was found to be a low yield but relative stable host for propagating the mKUZ cDNA. Based on these experiments, a chimera mKUZ-Fc, in which the catalytic domain of mKUZ is fused with human IgG Fc fragment, will be expressed and purified instead of the full length mKUZ.

Varies attempts to generate mammary epithelial cell lines stably expressing mKUZ and mDNK have failed. The expression of mKUZ under CMV promoter has been found to be unstable in mouse mammary epithelial cells, therefore I have constructed and is testing the retroviral vectors which placed mKUZ under tetracycline inducible promoter. The viral bearing mKUZ and mDNK will be used to infect mouse mammary cell lines for planned *in vivo* experiments.

Figure legend

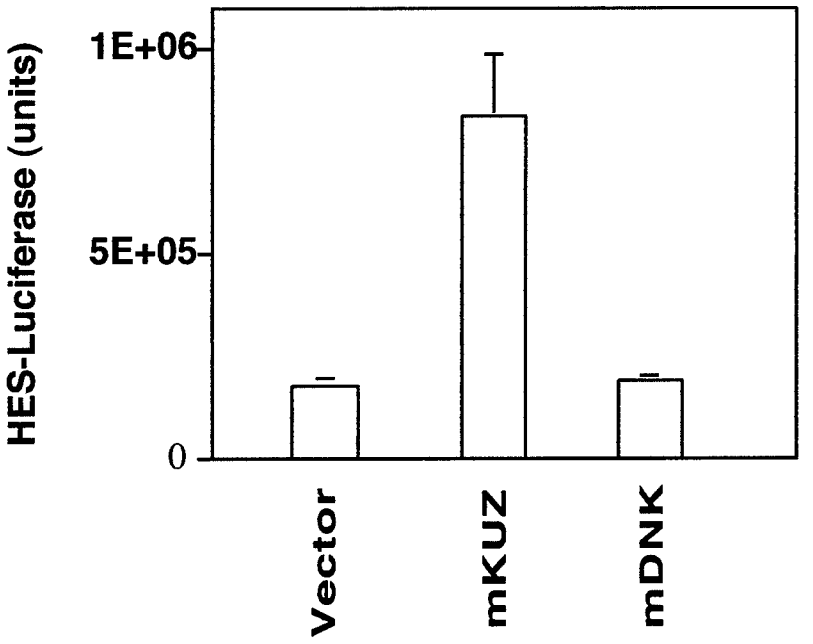
Figure 1. mKUZ activated Notch pathway. A) Expressing mKUZ, but not the dominant negative mKUZ (mDNK), induced HES transcription, which mimics the effects of Notch activation. B) Expressing mKUZ increased phosphorylated p38MAP kinase level in cells. COS-7 cells were transiently transfected, and incubated for 48 hours before the whole cell lysates were taken for measuring the luciferase activity and immunobloting with anti-phospho-p38 MAPK.

Figure 2. Cellular localization of mKUZ and its dominant negative form mDNK. COS-7 cells were transfected with vector (pcDNA) in A, mKUZ-Flag in B and mDNK-HA in C. 48 hours after transfection, cells were stained with biotin-conjugated M2 (anti-Flag) and 3F10 (anti-HA), followed by staining with Alex-488 streptavidin and Alex-594 anti-rat IgG.

Figure 3. Dominant negative form mKUZ (mDNK) down-regulated the wild type mKUZ in cells. COS-7 cells were transfected with vector alone (lane 1), mKUZ alone (lane 2), mKUZ plus mDNK (lane 3), and mDNK alone (lane 4). After 48 hours, cells were lysed in SDS sample buffer. Whole cell lysates were blotted onto membrane, probed with M2 (anti-Flag) antibody (up panel), stripped and reprobed with 12CA5 (anti-HA) antibody (low panel). Total DNA used in each transfection was made equal by adding pcDNA vector.

Figure 4. Dominant negative mKUZ rendered RAT1A cells resistant to N-myc induced transformation. RAT1A cells expressing N-myc-ER were transfected with vector (A and B), mKUZ (C and D), and mDNK (E and F). 24 hours later, ethanol (solvent control, 1 μl per ml) were added to A, C and E, whereas B, D and F were given 200 nM 4-Hydroxytamoxifen for 48 hours.

A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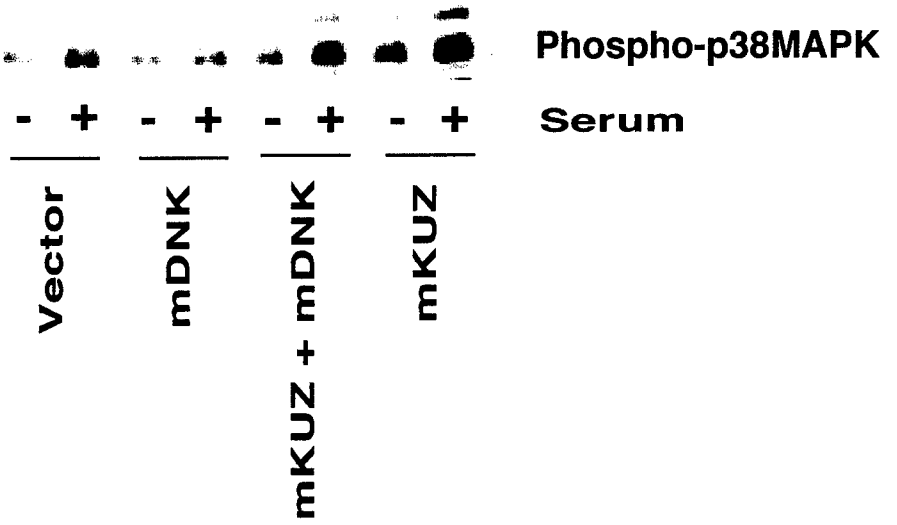
Vector
mKUZ
mDNK

B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mKUZ

Figure 1

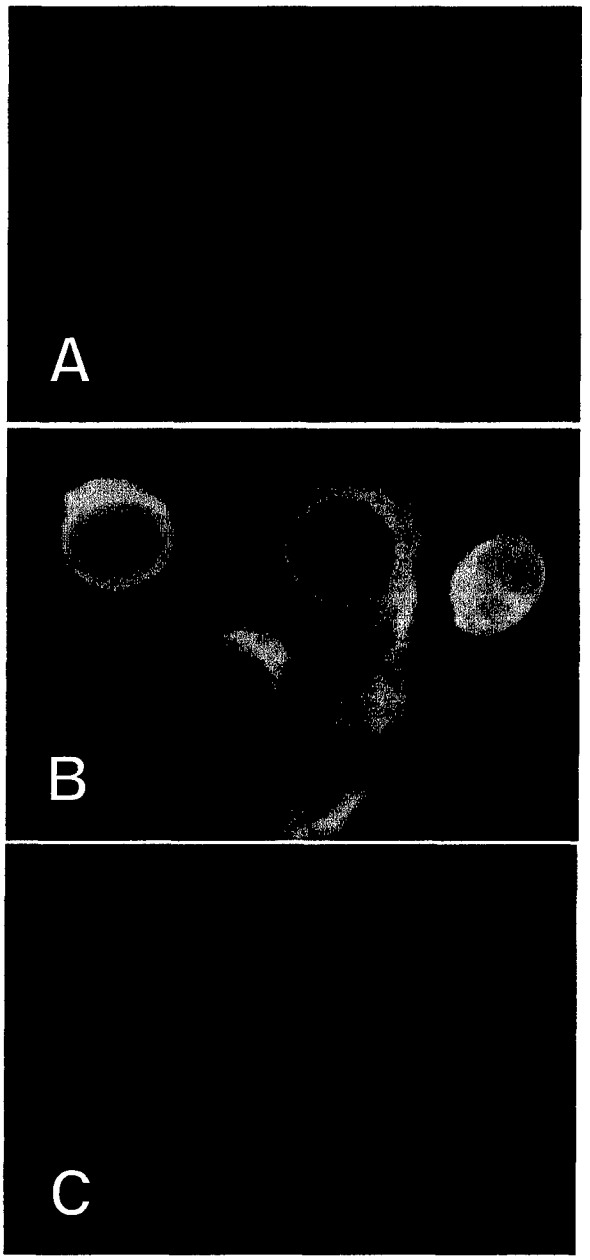


Figure 2

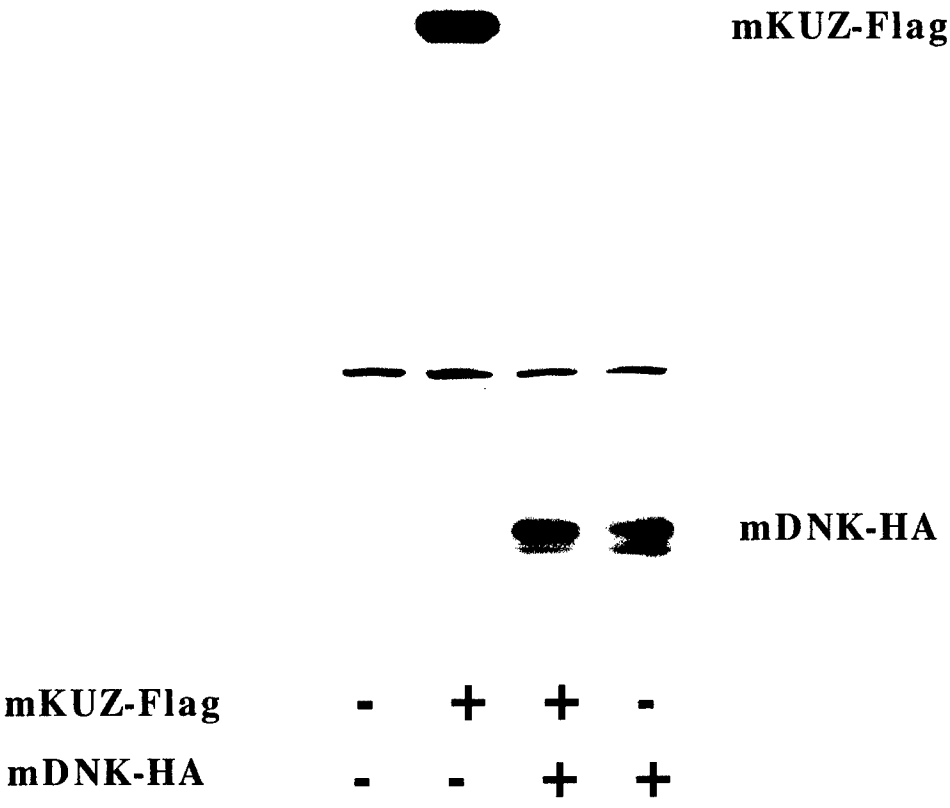


Figure 3

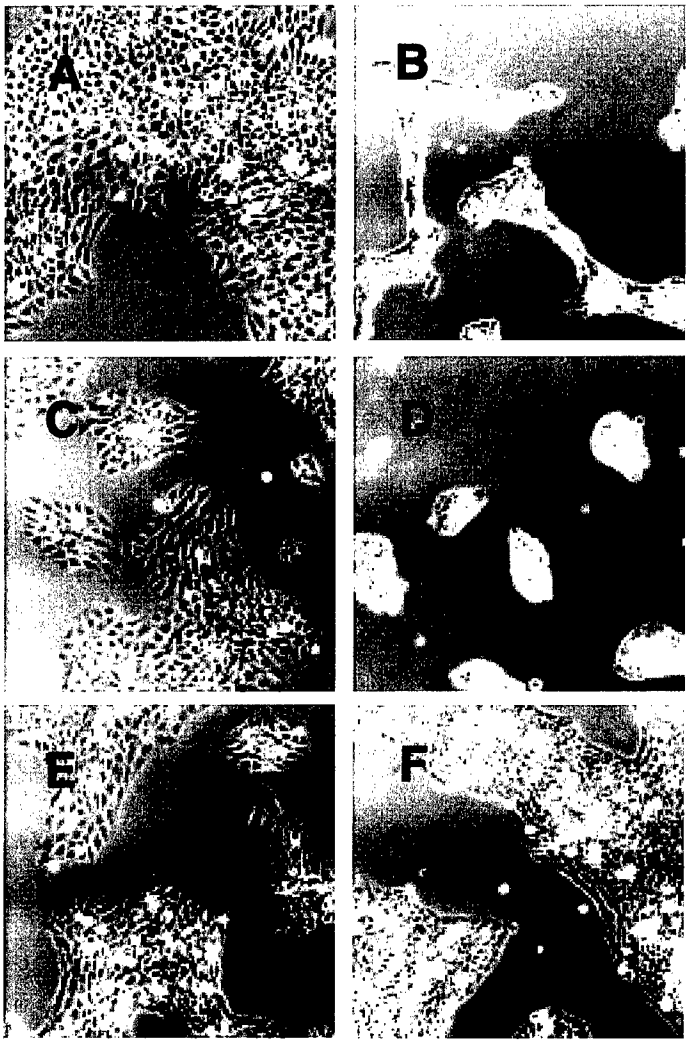


Figure 4

Note: This report contains unpublished data.

Training

During the last fiscal year, I have benefited very much by carring the proposed work in an excellent laboratory, by attending seminars, selected weekly from many serial or special seminars at UCSF including Cancer Center Seminars. I have also attended a Cold Spring Harbor meeting on the Proteases in Biology, which represents the most recent advances in the field.

In addition, I have requested and been granted to be a mentor in the Summer Research Program, a program designed to expose minority college senior to biomedical researches. My student Tajia van Hood, an African America women determined to be medical geneticist, is a McNeidar Scholar from University of California at Davis. By guiding her through the research project, I have learn many aspects of being a mentor for student pursuing advanced study, which are valuable experience for me to be an independent investigator.

Publication

Werb, Z and Yan, Y ***Science*** (1998) 282: 1279-1280 (attached)

Reprint Series
13 November 1998, Volume 282

SCIENCE

A Cellular Striptease Act

Zena Werb and Yibing Yan

A Cellular Striptease Act

Zena Werb and Yibing Yan

The cell surface is a dynamic place. During its life history the cell alters the repertoire of proteins displayed on its surface many times. Membrane-anchored adhesion molecules, receptors, ligands, and enzymes are removed and replaced as the cell proceeds through development and as its activation state changes. How is this wholesale refurnishing of the cell membrane orchestrated? One key mechanism is proteolytic processing of the ectodomain (extracellular domain) of such membrane proteins. Cleavage or shedding of the ectodomains of plasma membrane proteins—widely observed in cells in culture—is blocked by inhibitors of metalloproteinases (*1, 2*). This result suggests that transmembrane and soluble metalloproteinases, such as matrix metalloproteinases (MMPs) and their relatives, are rate-limiting for cleavage and shedding. Other evidence also implicates serine proteinases in these processing events (*3, 4*).

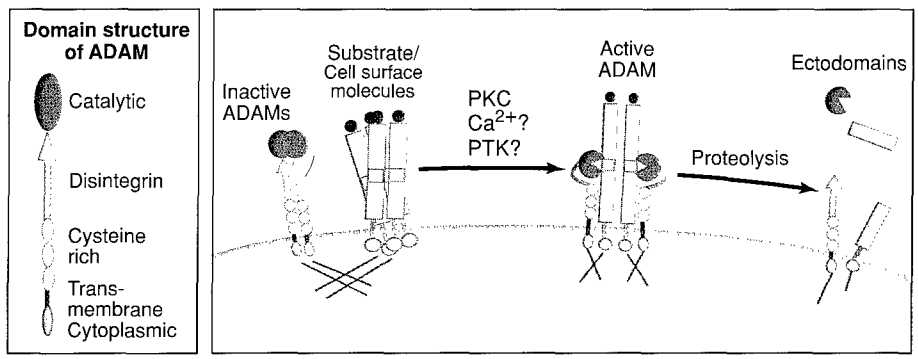
Enhanced online at www.sciencemag.org/cgi/content/full/282/5392/1279

The first such "sheddase" characterized was the tumor necrosis factor–α (TNF-α) converting enzyme (TACE) (*5*). The study by Peschon and colleagues (*6*) on page 1281of this issue now points to TACE's essential role in the shedding of ectodomains during mouse development. The surprise comes from the observation that mice lacking TACE do not show a phenotype indicative of a lack of TNF-α availability. Rather, they show the same phenotype as mice engineered to be without the epidermal growth factor (EGF) receptor—because TACE-mediated proteolysis makes available ligands for the EGF receptor, particularly transforming growth factor–α (TGF-α).

TACE turns out to be a membrane-anchored proteinase that is a member of the ADAM (a disintegrin and metalloproteinase) domain family of proteins that combines features of both cell surface adhesion molecules and proteinases (*8*). ADAMs all have a common domain organization, which endows these proteins with several potential functions—proteolysis, adhesion, signaling, and fusion (see figure below). The proteolytically competent ADAMs, such as TACE (ADAM17), are zinc-dependent metalloproteinases, closely related to the MMP family with which they share small molecule inhibitors and even one tissue inhibitor, TIMP-3 (*9, 10*). Several newly discovered MMPs appear to be hybrids of both MMP and ADAM domains (*11*), indicating that these two types of enzymes are part of one, larger family.

The ADAM proteinases are themselves targets of proteolytic events that ultimately strip off the catalytic domains (*5, 8*). This action could be a mechanism of functionally blunting the effects of the proteinases (see the figure on the next page). These soluble ADAMs may have proteolytic activity, as is the case for snake venom enzymes (*8*), but soluble TACE is much less active than membrane-bound enzyme (*5, 6*). The residual adhesive domains of ADAMs left after cleavage may have regulatory or adhesive functions. In support of this idea, a catalytic domain-deleted mutant of *Kuz* (ADAM10/SUP17), first identified as being required for cleavage of Notch during neural development in *Drosophila*, exerts a dominant negative effect (*8, 12*). During sperm maturation fertilin, a heterodimeric ADAM essential for sperm-egg interaction (*13*), also loses its catalytic domains by proteolytic processing. The remaining adhesive disintegrin domain is then competent to bind integrins.

How does TACE act? TACE is widely expressed in the animal. Mutation of the catalytic domain of TACE (*6*) reveals several distinct functions for this ADAM in development. Ligands for the EGF receptor, which is essential for epithelial development (*7*), are usually made and used locally (*14*). Although the growth factor precursors may have some biological activity (*15*), the new results imply that the membrane-anchored forms are essentially inactive precursors (*6*). TACE also cleaves ectodomains of other receptors and ligands, such as TNF-α, the p75 TNF receptor, and L-se-

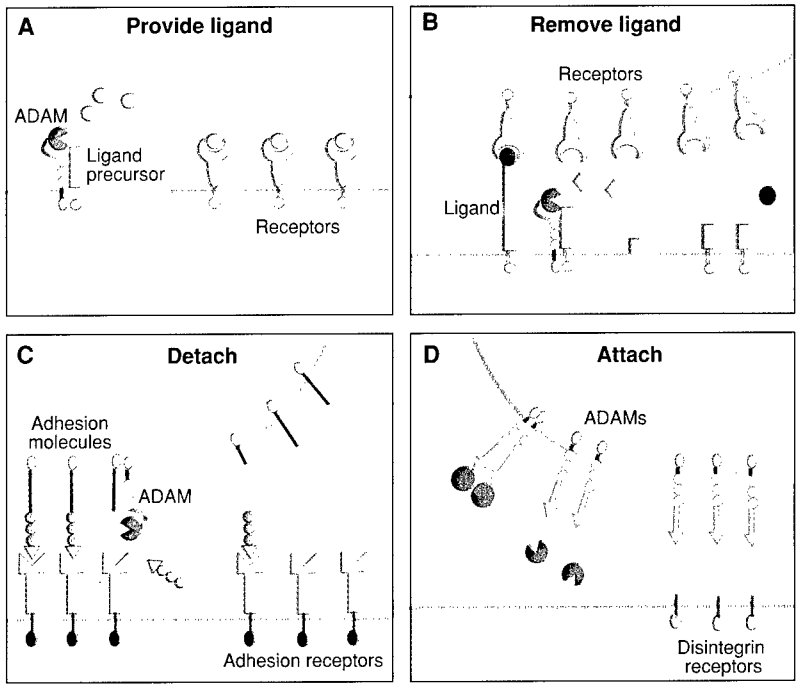


Activation of sheddases. The ADAM proteases (as dimers) and substrates are anchored apart in the plane of the membrane. Upon activation (via protein kinases and other pathways) they are brought together and proteolysis takes place, leading to free ectodomains.

The authors are at the Department of Anatomy, University of California, San Francisco, CA 94143-0452, USA. E mail: zena@itsa.ucsf.edu; yan@cgl.ucsf.edu

lectin, and thus participates in inflammatory and pathological reactions (*6*).

Processing membrane proteins by the ADAMs and other sheddases requires both the membrane-anchored enzyme and its substrate to be present in cis on the same cell (*6, 8, 12*). This presents several interesting problems. How are the active cell surface proteinases kept separate from their cell surface substrates until shedding is triggered? How do you exert selectivity for only certain ectodomain targets, out of many transmembrane proteins displayed on the cell surface? And how are the proteinase and substrate brought together in a coordinated manner so that all the cell surface substrate molecules can be removed within seconds, as occurs for the adhesion molecules L-selectin and syndecans (*4, 16*)?



Versatile shedding. Sheddases can supply or down-regulate ligands for receptors. Cleavage of adhesion molecules on cell surface or exposure of the disintegrin domain of ADAM regulate cell-cell and cell–extracellular matrix interactions.

Despite nonconserved cleavage sites that may be adjacent to the membrane or further out on the molecule, there are clues that a common strategy may operate in most cases. First, all ectodomain shedding is inhibited in a single mutant cell line (*1*). Second, the proteolysis is regulated in different cell types by activation of protein kinase C (PKC), calcium/calmodulin kinases, or receptor tyrosine kinases (*1, 17*). A model that accounts for these observations requires the processing proteinases and their transmembrane substrates both to be anchored in distinct domains of the plasma membrane, probably through cytoskeletal interactions (see figure on previous page). Upon cell activation, the attachments change and the proteinases and substrates become coclustered and can interact. Alternatively, the signaling cascade could modify the cytoplasmic domains of the proteinases or substrate, producing a conformational change that either activates the enzyme or makes the cleavage site available.

Although activation of the shedding reaction appears to control the rapid and complete removal of cell surface molecules such as L-selectin (an adhesion molecule involved in leukocyte rolling and extravasation into inflammatory sites) for most processing reactions there appears to be a constitutive level of ectodomain shedding. Processing is necessary to make available paracrine growth and survival factors such as TGF-α, EGF, HB-EGF, the kit ligand, and amphiregulin (*18*). This makes sense to allow for the consistent supply of growth factors (see figure above).

Endogenous inhibitors allow even finer control of the action of the shedding enzymes. Recently TACE was shown to be inhibited by TIMP-3, but not by the three other TIMPs that also inhibit MMPs (*10*). If TACE liberates a survival factor, then the presence of TIMP-3 could lead to cell death. This may explain why TIMP-3, but not other TIMPs, induce apoptosis (*19*).

Proteolysis of the ectodomains of growth factor coreceptors such as syndecan provide a second mechanism for regulating growth factor availability. Shedding the ectodomain of syndecan converts it to a potent inhibitor of FGF-2 (*20*). Just as shedding can make growth factor ligands available and control proliferation and survival, cleavage can also control cell death. Membrane-bound Fas ligand induces apoptosis by binding to the Fas receptor. Proteolysis functionally down-regulates the ligand and short-circuits apoptosis in lymphoid cell (*21*).

Cell surface adhesive molecules can also be regulated by proteolysis. An emerging paradigm is that cleavage of adhesive molecules not only alters adhesion, but completely revamps cell signaling. In the case of Notch, cleavage by *Kuz* is required to make it functional as a receptor, promoting adhesion, signaling, and cell lineage choices (*12*). Shedding of L-selectin by TACE or related enzymes inhibits leukocyte rolling and blunts their extravasation to inflammatory sites (*16*). The shedding of the ectodomains of E-cadherin (*22*) and transmembrane protein tyrosine phosphatases such as LAR have profound effects on cell-cell adhesions and also on important signaling pathways (*17*). These changing adhesion receptors and ligands may also be part of the apparatus for pathfinding in the nervous system.

Cells use a limited number of strategies to remodel their microenvironments. It is clear that the shedding process is an ancient, conserved, and fundamental pathway present from worms to humans. Thus, proteolysis by cell surface shedding enzymes provides a mechanism by which the wardrobe of externally displayed molecules can be changed or discarded. Spatial restriction of the enzymes and their substrates allows for either instant action or sustained activity.

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23. Supported by grants from NIH (CA72006, HD26732)

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ATTENTION OF:

MCMR-RMI-S (70-1y) 8 Jan 2003

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed. Request the limited distribution statement for the enclosed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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